A PROTEINASE INHIBITOR, PRECURSOR THEREOF AND GENETIC SEQUENCES ENCODING SAME

The present invention relates generally to proteinase inhibitors, a precursor thereof and to genetic sequences encoding same.

Nucleotide and amino acid sequences are referred to herein by sequence identity numbers (SEQ ID NOs) which are defined after the bibliography. A general summary of the SEQ ID NOs is provided before the examples.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Several members of the families Solanaceae and Fabaceae accumulate serine proteinase inhibitors in their storage organs and in leaves in response to wounding (Brown and Ryan, 1984; Richardson, 1977). The inhibitory activities of these proteins are directed against a wide range of proteinases of microbial and animal origin, but rarely against plant proteinases (Richardson, 1977). It is believed that these inhibitors are involved in protection of the plants against pathogens and predators. In potato tubers and legume seeds, the inhibitors can comprise 10% or more of the stored proteins (Richardson, 1977), while in leaves of tomato and potato (Green and Ryan, 1972), and alfalfa (Brown and Ryan, 1984), proteinase inhibitors can accumulate to levels of 2% of the soluble protein within 48 hours of insect attack, or other types of wounding (Brown & Ryan, 1984; Graham *et al*, 1986). High levels of these inhibitors (up to 50% of total soluble protein) are also present in unripe fruits of the wild tomato, *Lycopersicon peruvianum* (Pearce *et al*, 1988).

There are two families of serine proteinase inhibitors in tomato and potato (Ryan, 1984). Type I inhibitors are small proteins (monomer Mr 8100) which inhibit chymotrypsin at a single reactive site (Melville and Ryan, 1970; Plunkett *et al*, 1982). Inhibitors of the type II family generally contain two reactive sites, one of which inhibits chymotrypsin and the other trypsin (Bryant *et al*, 1976; Plunkett *et al*, 1982). The type II inhibitors have a monomer Mr of 12,300 (Plunkett *et al*, 1982). Proteinase inhibitor I accumulates in etiolated tobacco (*Nicotiana tabacum*) leaves (Kuo *et al*, 1984), and elicitors from *Phytophthora parasitica* var. *nicotianae* were found to induce proteinase inhibitor I accumulation in tobacco cell suspension cultures (Rickauer *et al*, 1989).

There is a need to identify other proteinase inhibitors and to investigate their potential use in the development of transgenic plants with enhanced protection against pathogens and predators. In accordance with the present invention, genetic sequences encoding a proteinase inhibitor precursor have been cloned. The precursor has multi-proteinase inhibitor domains and will be useful in developing a range of transgenic plants with enhanced proteinase inhibitor expression. Such plants will have enhanced protective properties against pathogens and predators. The genetic constructs of the present invention will also be useful in developing vaccines for ingestion by insects which are themselves predators or which act as hosts for plant pathogens. The recombinant precursor or monomeric inhibitors will also be useful in topical sprays and in assisting animals in feed digestion.

Accordingly, one aspect of the present invention relates to a nucleic acid molecule comprising a sequence of nucleotides which encodes or is complementary to a sequence which encodes a type II serine proteinase inhibitor (PI) precursor from a plant wherein said precursor comprises at least three PI monomers and wherein at least one of said monomers has a chymotrypsin specific site and at least one other of said monomers has a trypsin specific site.

The "nucleic acid molecule" of the present invention may be RNA or DNA (eg cDNA), single or double stranded and linear or covalently closed. The nucleic acid molecule may also be genomic DNA corresponding to the entire gene or a substantial portion thereof or to fragments or derivatives thereof. The nucleotide sequence may correspond to the naturally occurring nucleotide sequence of the genomic or cDNA clone or may contain single or multiple nucleotide substitutions, deletions and/or additions thereto. All such variants in the nucleic acid molecule either retain the ability to encode at least one monomer or active part thereof or are useful as hybridisation probes or polymerase chain reaction (PCR) primers for the same or similar genetic sequences in other sources.

Preferably, the PI precursor comprises at least four, more preferably at least five and even more preferably at least six PI monomers. Still more preferably, the PI precursor further comprises a signal sequence. The PI precursor of the present invention preferably comprises amino acid sequences which are process sites for cleavage into individual monomers.

The term "precursor" as used herein is not intended to place any limitation on the utility of the precursor molecule itself or a requirement that the molecule first be processed into monomers before PI activity is expressed. The precursor molecule has PI activity and the present invention is directed to the precursor and to the individual monomers of the precursor.

Furthermore, the present invention extends to a nucleic acid molecule comprising a sequence of nucleotides which encodes or is complementary to a sequence which encodes a hybrid type II serine PI precursor wherein said precursor comprises at least two monomers from different PIs. The at least two monomers may be modified such as being unable to be processed into individual monomers or may retain the ability to be so processed. Preferably, at least one of said monomers has a chymotrypsin specific site and the other of said monomers has a trypsin specific site. Preferably there are at least three monomers, more preferably at least four

monomers, still more preferably at least five monomers and yet still more preferably at least six monomers wherein at least two are from different PIs. In a most preferred embodiment, at least one of said monomers is a thionin. Such hybrid PI precursors and/or monomers thereof are particularly useful in generating molecules which are "multi-valent" in that they are active against a range of pathogens and predators such as both fungi and insects. Accordingly, reference herein to "PI precursor" includes reference to hybrid molecules.

The present invention is exemplified by the isolation of the subject nucleic acid molecule from *Nicotiana alata* which has the following nucleotide sequence (SEQ ID NO. 1) and a corresponding amino acid sequence (SEQ ID NO. 3):

```
                                                AAG GCT TGT ACC TTA AAC
                                                Lys Ala Cys Thr Leu Asn

TGT GAT CCA AGA ATT GCC TAT GGA GTT TGC CCG CGT TCA GAA GAA AAG
Cys Asp Pro Arg Ile Ala Tyr Gly Val Cys Pro Arg Ser Glu Glu Lys

AAG AAT GAT CGG ATA TGC ACC AAC TGT TGC GCA GGC ACG AAG GGT TGT
Lys Asn Asp Arg Ile Cys Thr Asn Cys Cys Ala Gly Thr Lys Gly Cys

AAG TAC TTC AGT GAT GAT GGA ACT TTT GTT TGT GAA GGA GAG TCT GAT
Lys Tyr Phe Ser Asp Asp Gly Thr Phe Val Cys Glu Gly Glu Ser Asp

CCT AGA AAT CCA AAG GCT TGT ACC TTA AAC TGT GAT CCA AGA ATT GCC
Pro Arg Asn Pro Lys Ala Cys Thr Leu Asn Cys Asp Pro Arg Ile Ala

TAT GGA GTT TGC CCG CGT TCA GAA GAA AAG AAG AAT GAT CGG ATA TGC
Tyr Gly Val Cys Pro Arg Ser Glu Glu Lys Lys Asn Asp Arg Ile Cys

ACC AAC TGT TGC GCA GGC ACG AAG GGT TGT AAG TAC TTC AGT GAT GAT
Thr Asn Cys Cys Ala Gly Thr Lys Gly Cys Lys Tyr Phe Ser Asp Asp

GGA ACT TTT GTT TGT GAA GGA GAG TCT GAT CCT AGA AAT CCA AAG GCT
Gly Thr Phe Val Cys Glu Gly Glu Ser Asp Pro Arg Asn Pro Lys Ala

TGT CCT CGG AAT TGC GAT CCA AGA ATT GCC TAT GGG ATT TGC CCA CTT
Cys Pro Arg Asn Cys Asp Pro Arg Ile Ala Tyr Gly Ile Cys Pro Leu
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GCA GAA GAA AAG AAG AAT GAT CGG ATA TGC ACC AAC TGT TGC GCA GGC
Ala Glu Glu Lys Lys Asn Asp Arg Ile Cys Thr Asn Cys Cys Ala Gly

AAA AAG GGT TGT AAG TAC TTT AGT GAT GAT GGA ACT TTT GTT TGT GAA
Lys Lys Gly Cys Lys Tyr Phe Ser Asp Asp Gly Thr Phe Val Cys Glu

GGA GAG TCT GAT CCT AAA AAT CCA AAG GCC TGT CCT CGG AAT TGT GAT
Gly Glu Ser Asp Pro Lys Asn Pro Lys Ala Cys Pro Arg Asn Cys Asp

GGA AGA ATT GCC TAT GGG ATT TGC CCA CTT TCA GAA GAA AAG AAG AAT
Gly Arg Ile Ala Tyr Gly Ile Cys Pro Leu Ser Glu Glu Lys Lys Asn

GAT CGG ATA TGC ACC AAC TGC TGC GCA GGC AAA AAG GGT TGT AAG TAC
Asp Arg Ile Cys Thr Asn Cys Cys Ala Gly Lys Lys Gly Cys Lys Tyr

TTT AGT GAT GAT GGA ACT TTT GTT TGT GAA GGA GAG TCT GAT CCT AAA
Phe Ser Asp Asp Gly Thr Phe Val Cys Glu Gly Glu Ser Asp Pro Lys

AAT CCA AAG GCT TGT CCT CGG AAT TGT GAT GGA AGA ATT GCC TAT GGG
Asn Pro Lys Ala Cys Pro Arg Asn Cys Asp Gly Arg Ile Ala Tyr Gly

ATT TGC CCA CTT TCA GAA GAA AAG AAG AAT GAT CGG ATA TGC ACA AAC
Ile Cys Pro Leu Ser Glu Glu Lys Lys Asn Asp Arg Ile Cys Thr Asn

TGT TGC GCA GGC AAA AAG GGC TGT AAG TAC TTT AGT GAT GAT GGA ACT
Cys Cys Ala Gly Lys Lys Gly Cys Lys Tyr Phe Ser Asp Asp Gly Thr

TTT GTT TGT GAA GGA GAG TCT GAT CCT AGA AAT CCA AAG GCC TGT CCT
Phe Val Cys Glu Gly Glu Ser Asp Pro Arg Asn Pro Lys Ala Cys Pro

CGG AAT TGT GAT GGA AGA ATT GCC TAT GGA ATT TGC CCA CTT TCA GAA
Arg Asn Cys Asp Gly Arg Ile Ala Tyr Gly Ile Cys Pro Leu Ser Glu

GAA AAG AAG AAT GAT CGG ATA TGC ACC AAT TGT TGC GCA GGC AAG AAG
Glu Lys Lys Asn Asp Arg Ile Cys Thr Asn Cys Cys Ala Gly Lys Lys

GGC TGT AAG TAC TTT AGT GAT GAT GGA ACT TTT ATT TGT GAA GGA GAA
Gly Cys Lys Tyr Phe Ser Asp Asp Gly Thr Phe Ile Cys Glu Gly Glu

TCT GAA TAT GCC AGC AAA GTG GAT GAA TAT GTT GGT GAA GTG GAG AAT
Ser Glu Tyr Ala Ser Lys Val Asp Glu Tyr Val Gly Glu Val Glu Asn

GAT CTC CAG AAG TCT AAG GTT GCT GTT TCC
Asp Leu Gln Lys Ser Lys Val Ala Val Ser

This is done, however, with the understanding that the present invention extends to an equivalent or substantially similar nucleic acid molecule from any other plant. By "equivalent" and "substantially similar" is meant at the level of nucleotide sequence, amino acid sequence, antibody reactivity, monomer composition and/or processing of the precursor to produce monomers. For example, a nucleotide sequence having a percentage sequence similarity of at least 55%, such as about 60-65%, 70-75%, 80-85% and over 90% when compared to the sequence of SEQ ID NO. 1 would be considered "substantially similar" to the subject nucleic acid molecule provided that such a substantially similar sequence encodes a PI precursor having at least three monomers and preferably four, five or six monomers as hereinbefore described.

In a particularly preferred embodiment, the nucleic acid molecule further encodes a signal sequence 5′ to the open reading frame and/or a nucleotide sequence 3′ of the coding region providing a full nucleotide sequence as follows (SEQ ID NO. 2):

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CGAGTAAGTA TGGCTGTTCA CAGAGTTAGT TTCCTTGCTC TCCTCCTCTT ATTTGGAATG

TCTCTGCTTG TAAGCAATGT GGAACATGCA GATGCC AAG GCT TGT ACC TTA AAC
                                        Lys Ala Cys Thr Leu Asn

TGT GAT CCA AGA ATT GCC TAT GGA GTT TGC CCG CGT TCA GAA GAA AAG
Cys Asp Pro Arg Ile Ala Tyr Gly Val Cys Pro Arg Ser Glu Glu Lys

AAG AAT GAT CGG ATA TGC ACC AAC TGT TGC GCA GGC ACG AAG GGT TGT
Lys Asn Asp Arg Ile Cys Thr Asn Cys Cys Ala Gly Thr Lys Gly Cys

AAG TAC TTC AGT GAT GAT GGA ACT TTT GTT TGT GAA GGA GAG TCT GAT
Lys Tyr Phe Ser Asp Asp Gly Thr Phe Val Cys Glu Gly Glu Ser Asp

CCT AGA AAT CCA AAG GCT TGT ACC TTA AAC TGT GAT CCA AGA ATT GCC
Pro Arg Asn Pro Lys Ala Cys Thr Leu Asn Cys Asp Pro Arg Ile Ala

TAT GGA GTT TGC CCG CGT TCA GAA GAA AAG AAG AAT GAT CGG ATA TGC
Tyr Gly Val Cys Pro Arg Ser Glu Glu Lys Lys Asn Asp Arg Ile Cys

ACC AAC TGT TGC GCA GGC ACG AAG GGT TGT AAG TAC TTC AGT GAT GAT
Thr Asn Cys Cys Ala Gly Thr Lys Gly Cys Lys Tyr Phe Ser Asp Asp
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GGA ACT TTT GTT TGT GAA GGA GAG TCT GAT CCT AGA AAT CCA AAG GCT
Gly Thr Phe Val Cys Glu Gly Glu Ser Asp Pro Arg Asn Pro Lys Ala

TGT CCT CGG AAT TGC GAT CCA AGA ATT GCC TAT GGG ATT TGC CCA CTT
Cys Pro Arg Asn Cys Asp Pro Arg Ile Ala Tyr Gly Ile Cys Pro Leu

GCA GAA GAA AAG AAG AAT GAT CGG ATA TGC ACC AAC TGT TGC GCA GGC
Ala Glu Glu Lys Lys Asn Asp Arg Ile Cys Thr Asn Cys Cys Ala Gly

AAA AAG GGT TGT AAG TAC TTT AGT GAT GAT GGA ACT TTT GTT TGT GAA
Lys Lys Gly Cys Lys Tyr Phe Ser Asp Asp Gly Thr Phe Val Cys Glu

GGA GAG TCT GAT CCT AAA AAT CCA AAG GCC TGT CCT CGG AAT TGT GAT
Gly Glu Ser Asp Pro Lys Asn Pro Lys Ala Cys Pro Arg Asn Cys Asp

GGA AGA ATT GCC TAT GGG ATT TGC CCA CTT TCA GAA GAA AAG AAG AAT
Gly Arg Ile Ala Tyr Gly Ile Cys Pro Leu Ser Glu Glu Lys Lys Asn

GAT CGG ATA TGC ACC AAC TGC TGC GCA GGC AAA AAG GGT TGT AAG TAC
Asp Arg Ile Cys Thr Asn Cys Cys Ala Gly Lys Lys Gly Cys Lys Tyr

TTT AGT GAT GAT GGA ACT TTT GTT TGT GAA GGA GAG TCT GAT CCT AAA
Phe Ser Asp Asp Gly Thr Phe Val Cys Glu Gly Glu Ser Asp Pro Lys

AAT CCA AAG GCT TGT CCT CGG AAT TGT GAT GGA AGA ATT GCC TAT GGG
Asn Pro Lys Ala Cys Pro Arg Asn Cys Asp Gly Arg Ile Ala Tyr Gly

ATT TGC CCA CTT TCA GAA GAA AAG AAG AAT GAT CGG ATA TGC ACA AAC
Ile Cys Pro Leu Ser Glu Glu Lys Lys Asn Asp Arg Ile Cys Thr Asn

TGT TGC GCA GGC AAA AAG GGC TGT AAG TAC TTT AGT GAT GAT GGA ACT
Cys Cys Ala Gly Lys Lys Gly Cys Lys Tyr Phe Ser Asp Asp Gly Thr

TTT GTT TGT GAA GGA GAG TCT GAT CCT AGA AAT CCA AAG GCC TGT CCT
Phe Val Cys Glu Gly Glu Ser Asp Pro Arg Asn Pro Lys Ala Cys Pro

CGG AAT TGT GAT GGA AGA ATT GCC TAT GGA ATT TGC CCA CTT TCA GAA
Arg Asn Cys Asp Gly Arg Ile Ala Tyr Gly Ile Cys Pro Leu Ser Glu

GAA AAG AAG AAT GAT CGG ATA TGC ACC AAT TGT TGC GCA GGC AAG AAG
Glu Lys Lys Asn Asp Arg Ile Cys Thr Asn Cys Cys Ala Gly Lys Lys

GGC TGT AAG TAC TTT AGT GAT GAT GGA ACT TTT ATT TGT GAA GGA GAA
Gly Cys Lys Tyr Phe Ser Asp Asp Gly Thr Phe Ile Cys Glu Gly Glu

TCT GAA TAT GCC AGC AAA GTG GAT GAA TAT GTT GGT GAA GTG GAG AAT
Ser Glu Tyr Ala Ser Lys Val Asp Glu Tyr Val Gly Glu Val Glu Asn

GAT CTC CAG AAG TCT AAG GTT GCT GTT TCC TAAGTCCTAA CTAATAATAT
Asp Leu Gln Lys Ser Lys Val Ala Val Ser

GTAGTCTATG TATGAAACAA AGGCATGCCA ATATGCTCTG TCTTGCCTGT AATCTGTAAT

ATGGTAGTGG AGCTTTTCCA CTGCCTGTTT AATAAGAAAT GGAGCACTAG TTTGTTTTAG

TTAAAAAAAA AAAAAAAAAA

including substantially similar variants thereof.

Accordingly, a preferred embodiment of the present invention provides a nucleic acid molecule comprising a sequence of nucleotides as set forth in SEQ ID NO. 1 or 2 which encodes or is complementary to a sequence which encodes a type II serine PI precursor from *Nicotiana alata* or having at least 55% similarity to said precursor or at least one domain therein wherein said precursor comprises a signal peptide and at least five monomers and wherein one of said monomers has a chymotrypsin specific site and four of said monomers have trypsin specific sites.

In still a more preferred embodiment, the nucleic acid molecule is a cDNA molecule and comprises a nucleotide sequence generally as set forth in SEQ ID NO. 1 or 2 or being substantially similar thereto as hereinbefore defined to the whole of said sequence or to a domain thereof.

Another aspect of the present invention is directed to a nucleic acid molecule comprising a sequence of nucleotides which encodes or is complementary to a sequence which encodes a single type II serine PI having either a chymotrypsin specific site or a trypsin specific site and wherein said PI is a monomer of a precursor PI having at least three monomers of which at least one of said monomers has a chymotrypsin site and the other of said monomers has a trypsin site. Preferably, however, the precursor has four, five or six monomers and is as hereinbefore defined.

In its most preferred embodiment, the plant is *N. alata* (Link et Otto) having self-incompatibility genotype S_1S_3, S_3S_3 or S_6S_6, and the nucleic acid molecule is isolatable from or complementary to genetic sequences isolatable from stigmas and styles of mature plants. The corresponding mRNA is approximately 1.4 kb and the cDNA has six conserved domains wherein the first two domains are 100% identical and contain chymotrypsin-specific sites (Leu-Asn). The third, fourth and fifth domains share 95-98% identity and have sites specific for trypsin (Arg-Asn). A sixth domain which also has a trypsin specific site has less identity to the third, fourth and fifth domains (79-90%) due mainly to a divergent 3' sequence (see Table 1). The preferred PI inhibitor of the present invention has a molecular weight of approximately 42-45 kDa with an approximately 29 amino acid signal sequence.

The N-terminal sequence of the monomeric PI is represented in each of the six repeated domains in the predicted sequence of the PI precursor protein. Thus, it is likely that the PI precursor protein is cleaved at six sites to produce seven peptides. Six of the seven peptides, peptides 2, 3, 4, 5, 6 and 7 (Figure 1, residues 25-82 [SEQ ID NO. 5], 83-140 [SEQ ID NO. 6], 141-198 [SEQ ID NO. 7], 199-256 [SEQ ID NO. 8], 257-314 [SEQ ID NO. 9] and 315-368 [SEQ ID NO. 9], respectively), would be in the same molecular weight range as the monomeric PI (about 6 kDa) and would have the same N-terminal sequence. Peptide 7 does not contain a consensus site for trypsin or chymotrypsin. Peptide 1 (residues 1-24 [SEQ ID NO. 4], Figure 1) is smaller than 6 kD, has a different N-terminus and was not detected in a purified monomeric PI preparation. It could be envisaged that peptide 1 and peptide 7 would form a functional proteinase inhibitor with the inhibitory site on peptide 1 held in the correct conformation by disulphide bonds formed between the two peptides.

Although not intending to limit the present invention to any one hypothesis, the PI precursor may be processed by a protease responsible, for example, for cleavage of an Asn-Asp linkage, to produce the bioactive monomers. More particularly, the protease sensitive sequence is R_1-X_1-X_2-Asn-Asp-R_2 where R_1, R_2, X_1 and X_2 are

defined below. The discovery of such a sequence will enable the engineering of peptides and polypeptides capable of being processed in a plant by cleavage of the protease sensitive sequence. According to this aspect of the present invention there is provided a protease sensitive peptide comprising the amino acid sequence:

$$-X_1-X_2-Asn-Asp-$$

wherein X_1 and X_2 are any amino acid but are preferably both Lys residues. The protease sensitive peptide may also be represented as:

$$R_1-X_1-X_2-Asn-Asp-R_2$$

wherein X_1 and X_2 are preferably the same and are preferably both Lys residues and wherein R_1 and R_2 are the same or different, any D or L amino acid, a peptide, a polypeptide, a protein, or a non-amino acid moiety or molecule such as, but not limited to, an alkyl (eg methyl, ethyl), substituted alkyl, alkenyl, substituted alkenyl, acyl, dienyl, arylalkyl, arylalkenyl, aryl, substituted aryl, heterocyclic, substituted heterocyclic, cycloalkyl, substituted cycloalkyl, halo (e.g. Cl, Br, I, F), haloalkyl, nitro, hydroxy, thiol, sulfonyl, carboxy, alkoxy, aryloxy and alkylaryloxy group and the like as would be apparent to one skilled in the art. By alkyl, substitued alkyl, alkenyl and substituted alkenyl and the like is meant to encompass straight and branched molecules, lower (C_1 - C_6) and higher (more than C_6) derivatives. The term "substituted" includes all the substituents set forth above.

In its most preferred embodiment, the protease sensitive peptide is:

$$R_1-X_1-X_2-Asn-Asp-R_2$$

wherein R_1 and R_2 are the same or different and are peptides or polypeptides and wherein X_1 and X_2 are both Lys residues.

Such a protease sensitive peptide can be placed between the same or different monomers so that upon expression in a suitable host or *in vitro* the larger molecule can be processed to the peptides located between the protease sensitive peptides.

The present invention also extends to a nucleic acid molecule comprising a sequence of nucleotides which encodes or is complementary to a sequence which encodes a protease sensitive peptide comprising the sequence:

sub B2

$$-X_1-X_2-Asn-Asp-$$

wherein X_1 and X_2 are preferably the same and are most preferably both Lys residues. Such a nucleic acid molecule may be part of a larger nucleotide sequence encoding, for example, a precursor polypeptide capable of being processed via the protease sensitive sequence into individual peptides or monomers.

The protease sensitive peptide of the present invention is particularly useful in generating poly and/or multi-valent "precursors" wherein each monomer is the same or different and directed to the same or different activities such as anti-viral, anti-bacterial, anti-fungal, anti-pathogen and/or anti-predator activity.

Although not wishing to limit this aspect of the invention to any one hypothesis or proposed mechanism of action, it is believed that the protease acts adjacent the Asn residue as more particularly between the Asn-Asp residues.

The present invention extends to an isolated type II serine PI precursor from a plant wherein said precursor comprises at least three PI monomers and wherein at least one of said monomers has a chymotrypsin specific site and at least one other of said monomers has a trypsin specific site. Preferably, the PI precursor has four, five or six monomers and is encoded by the nucleic acid molecule as hereinbefore described. The present invention also extends to the individual monomers comprising the precursor. The present invention also extends to a hybrid recombinant PI precursor

molecule comprising at least two monomers from different PIs as hereinbefore described.

The isolated PI or PI precursor may be in recombinant form and/or biologically pure. By "biologically pure" is meant a preparation of PI, PI precursor and/or any mixtures thereof having undergone at least one purification step including ammonium sulphate precipitation, Sephadex chromatography and/or affinity chromatography. Preferably, the preparation comprises at least 20% of the PI, PI precursor or mixture thereof as determined by weight, activity antibody, reactivity and/or amino acid content. Even more preferably, the preparation comprises 30-40%, 50-60% or at least 80-90% of PI, PI precursor or mixture thereof.

The PI or its precursor may be naturally occurring or be a variant as encoded by the nucleic acid variants referred to above. It may also contain single or multiple substitutions, deletions and/or additions to its amino acid sequence or to non-proteinaceous components such as carbohydrate and/or lipid moieties.

The recombinant and isolated PI, PI precursor and mixtures thereof are useful as laboratory reagents, in the generation of antibodies, in topically applied insecticides as well as orally ingested insecticides.

The recombinant PI or PI precursor may be provided as an insecticide alone or in combination with one or more carriers or other insecticides such as the BT crystal protein.

The PI of the present invention is considered to have a defensive role in organs of the plant, for example, the stigma, against the growth or infection by pests and pathogens such as fungi, bacteria and insects. There is a need, therefore, to develop genetic constructs which can be used to generate transgenic plants capable of expressing the PI precursor where this can be processed into monomers of a monomeric PI itself.

Accordingly, another aspect of the present invention contemplates a genetic construct comprising a nucleic acid molecule comprising a sequence of nucleotides which encodes or is complementary to a sequence which encodes a type II serine PI precursor or monomer thereof from a plant wherein said precursor comprises at least three PI monomers and wherein at least one of said monomers has a chymotrypsin specific site and at least one of said other monomers has a trypsin specific site and said genetic sequence further comprises expression means to permit expression of said nucleic acid molecule, replication means to permit replication in a plant cell or, alternatively, integration means, to permit stable integration of said nucleic acid molecule into a plant cell genome. Preferably, the expression is regulated such as developmentally or in response to infection such as being regulated by an existing PI regulatory sequence. Preferably, the expression of the nucleic acid molecule is enhanced to thereby provide greater endogenous levels of PI relative to the levels in the naturally occurring plant. Alternatively, the PI precursor cDNA of the present invention is usable to obtain a promoter sequence which can then be used in the genetic construct or to cause its manipulation to thereby permit over-expression of the equivalent endogenous promoter. In another embodiment the PI precursor is a hybrid molecule as hereinbefore described.

Yet another aspect of the present invention is directed to a transgenic plant carrying the genetic sequence and/or nucleic acid molecule as hereinbefore described and capable of producing elevated, enhanced or more rapidly produced levels of PI and/or PI precursor or hybrid PI precursor when required. Preferably, the plant is a crop plant or a tobacco plant but other plants are usable where the PI or PI precursor nucleic acid molecule is expressable in said plant. Where the transgenic plant produces PI precursor, the plant may or may not further process the precursor into monomers. Alternatively, the genetic sequence may be part of a viral or bacterial vector for transmission to an insect to thereby control pathogens in insects which would consequently interrupt the transmission of the pathogens to plants.

In still yet another aspect of the present invention, there is provided antibodies to the PI precursor or one or more of its monomers. Antibodies may be monoclonal or polyclonal and are useful in screening for PI or PI precursor clones in an expression library or for purifying PI or PI precursor in a fermentation fluid, supernatant fluid or plant extract.

The genetic constructs of the present invention can also be used to populate the gut of insects to act against the insect itself or any plant pathogens therein or to incorporate into the gut of animals to facilitate the digestion of plant material.

The present invention is further described by reference to the following non-limiting Figures and Examples.

In the Figures:

Figure 1 shows the nucleic acid sequence (SEQ ID NO. 2) of the pNA-PI-2 insert and the corresponding amino acid sequence (SEQ ID NO. 3) of the *N. alata* PI protein. The amino acid sequence is numbered beginning with 1 for the first amino acid of the mature protein. The signal sequence is encoded by nucleotides 1 to 97 and the amino acid residues have been assigned negative numbers. The reactive site residues of the inhibitor are boxed. The *N. alata* PI sequence contains six similar domains (domain 1, residues 1 to 58, domain 2, residues 59-116, domain 3, residues 117-174, domain 4, residues 175-232, domain 5, residues 233-290 and domain 6, residues 291-343).

Figure 2 is a photographic representation showing a gel blot analysis of RNA from various organs of *N. alata*. Gel Blot of RNA isolated from organs of *N. alata* and from stigmas and styles of *N. tabacum* and *N. sylvestris*, hybridised with the cDNA clone NA-PI-2. St, stigma and style; Ov, ovaries; Po, pollen; Pe, petals; Se, sepals; L, non-wounded leaves; L4, leaves 4h after wounding; L24, leaves 24h after wounding; Nt, *N. tabacum* stigma and style; Ns, *N. sylvestris* stigma and style; Na

*Hin*dIII restriction fragments of Lambda-DNA.

The NA-PI-2 clone hybridised to 2 mRNA species (1.0 and 1.4kb). The larger mRNA was predominant in stigma and styles, whereas the smaller mRNA species was more dominant in other tissues. After high stringency washes, the 1.0kb mRNA from stigma and style no longer hybridises to the NA-PI-2 probe.

Figure 3 is a photographic presentation depicting *in situ* localisation of RNA homologous to NA-PI-2 in stigma and style.

(a) Autoradiograph of a longitudinal cryosection through the stigma and style of a 1cm long bud after hybridisation with the ^{32}P-labelled NA-PI-2 cDNA probe.

(b) The same section as (a), stained with toluidine blue. c, cortex; v, vascular bundles; tt, transmitting tract; s, stigmatic tissue.

The cDNA probe labelled the cells of the stigma heavily and some hybridisation to the vascular bundles can be seen. There was no hybridisation to the epidermis, cortical tissue or transmitting tissue. Scale bars = 200 μm.

Figure 4 is a photographic representation of a gel blot analysis of genomic DNA of *N. alata*. Gel blot analysis of *N. alata* genomic DNA digested with the restriction enzymes *Eco*RI or *Hin*dIII, and probed with radiolabelled NA-PI-2. Size markers (kb) are *Hin*dIII restriction fragments of Lambda-DNA.

*Eco*RI produced two hybridising fragments (11kb and 7.8kb), while *Hin*dIII gave three large hybridising fragments (16.6, 13.5 and 10.5kb). The NA-PI-2 clone appears to belong to a small multigene family consisting of at least two members.

Figure 5 is a graphic representation of PI activity in various organs of *N. alata*. Buffer soluble extracts from various organs were tested for their ability to inhibit trypsin and chymotrypsin. Stigma and sepal extracts were the most effective inhibitors of both trypsin (A) and chymotrypsin (B).

Figure 6 depicts the steps of the purification of PI from *N. alata* stigmas.

(a) Sephadex G-50 gel filtration chromatography of ammonium sulphate precipitated proteins from stigma extracts. The PI activity eluted late in the profile.

(b) 20% w/v SDS-polyacrylamide gel (Laemmli, 1970) of fractions across the gel filtration column. The gel was silver stained and molecular weight markers (Pharmacia peptide markers) are in kilodaltons. A protein of about 6kD (arrowed) coelutes with the proteinase inhibitor activity.

(c) Analysis of PI-containing fractions at different stages of the purification procedure, by SDS-PAGE. Lane 1, crude stigma extract (5μg); Lane 2, stigma proteins precipitated by 80% w/v ammonium sulphate (5μg); Lane 3, PI protein eluted from the chymotrypsin affinity column (1μg).

The PI is a 6kD protein and is a major component in unfractionated buffer soluble extracts from stigmas.

Figure 7 is a graphical representation showing hydropathy plots of the PI proteins encoded by the NA-PI-2 clone from *N. alata* and the potato and tomato PI II cDNAs. Values above the line denote hydrophobic regions and values below the line denote hydrophilic regions. The putative signal peptides are shaded. The hydrophobicity profile was generated using the predictive rules of Kyte and Doolittle (1982) and a span of 9 consecutive amino acids.

(a) **Hydropathy profile of the *N. alata* PI protein.** The six repeated domains in the predicted precursor protein are labelled I-VI. The hydrophilic regions containing the putative cleavage sites for production of the 6kD PI species are arrowed. The regions corresponding to the peptides that would be produced by cleavage at these sites are marked C for chymotrypsin inhibitor, T for trypsin inhibitor and x for the two flanking peptides.

(b) **Hydropathy profile of the potato PI II protein.** (Sanchez-Serrano *et al*, 1986). The two repeated domains in the PI II protein are labelled I and II. The putative cleavage sites for production of PCI-1 are arrowed (Hass *et al*, 1982) and the region spanned by PCI-1 is marked.

(c) Hydropathy profile of the polypeptide encoded by the tomato PI II cDNA (Graham *et al*, 1985). The two domains are labelled, I and II and the residues which would be potential processing sites are arrowed. These sites are not present in regions predicted to be hydrophilic and consequently a cleavage product is not marked.

Figure 8 shows an immunoblot analysis of the PI protein in stigmas of developing flowers.

(a) Developing flowers of *N. alata*.

(b) SDS-PAGE of stigma proteins at the stages of development shown in (a) 5 µg of each extract was loaded. The peptide gel was silver stained and molecular weight markers (LKB Low Molecular weight and Pharmacia peptide markers) are in kilodaltons.

(c) Immunoblot of a gel identical to (b), probed with anti-PI antiserum.

Stigmas from developing flowers contain four proteins of approximately 42kD, 32kD, 18kD and 6kD that bind to the anti-PI antibody. The 42kD and the 18kD components decrease in concentration as the flowers mature, while the 6kD PI protein reaches a maximum concentration just before anthesis. The level of the 32kD component, which runs as a doublet, does not alter significantly during flower development.

Figure 9 shows the separation and identification of the 6kD proteinase inhibitor species from *N.alata* stigmas

A. Separation of the 6kD PIs by reversed phase HPLC chromatography

Four major peaks were obtained with retention times of about 15.5min(peak1), 20.5min(peak2), 22.5min(peak3), 24min(peak4). The peptides in each peak have been identified by a combination of N-terminal analysis and mass spectrometry. See B for description of C1 and T1-T4.

B. The five homologous peptides produced from the PI precursor protein: C1, chymotrypsin inhibitor, T1-T4 trypsin inhibitors. The solid bars represent the reactive sites of the inhibitors. The precursor protein is drawn minus the signal sequence. region of the six repeated domains (amino acids 1-343, Fig.1). non-repeated sequence (amino acids 344-368, Fig.1). The arrows point to the processing sites in the precursor protein.

C. The amino acid sequence of C1 and T1-T4 predicted from the cDNA clone and confirmed by N-terminal sequencing of the purified peptides. The amino acid at the carboxy-terminus of each peptide was obtained by accurate mass determination using an electro-spray mass spectrometer. The C1 and T1 inhibitors differ by five amino acids (bold). Two of these amino acids are located at the reactive site (underlined) and the other two to three reside at the carboxy-terminus. Peptides T2-T4 have changes in three amino acids (boxed) that are conserved between C1 and T1. Peptides T2 and T3 are identical to each other. Mass spectrometry was used to demonstrate that other forms of C1 and T1-T4 occur due to non-precise trimming at the N- and C-termini. That is, some forms are missing residue 1 or residue 53 and others are missing both residue 1 and 53 (see Table 2).

Figure 10 shows the amino-acid sequence around the processing sites in the precursor PI protein.

The sequence in bold is the amino-terminal sequence obtained from the purified PI protein. The sequence labelled with negative numbers is the flanking sequence predicted from the cDNA clone. The predicted precursor protein contains six repeats of this sequence.

Figure 11 shows the PI precursor produced in a baculovirus expression system and the products obtained after digestion of the affinity purified PI precursor by the endoproteinase Asp-N.

A. The PI precursor produced by the recombinant baculovirus.

Immunoblot containing affinity and HPLC purified PI precursor from *N.alata* stigmas at the green bud stage of development (lane 1) and affinity purified PI precursor produced by the recombinant baculovirus (lane 2). Proteins were fractionated by electrophoresis on a 15% w/v SDS-polyacrylamide gel prior to electrophoretic transfer to nitrocellulose. The blot was incubated with the antibody raised in rabbits to the 6kD PI species from stigmas. The recombinant virus produced an immunorective protein of 42kD that is the same size as the PI precursor protein produced by stigmas (arrowed).

B. Cleavage of the PI precursor by endoproteinase Asp-N.

15% SDS-polyacrylamide gel stained with silver containing: 1, PI precursor, produced by baculovirus, incubated without enzyme. 2, enzyme incubated without precursor. 6kD, PI peptides of about 6kD purified from *N.alata* stigmas. 1m, 5m, 30m, reaction products produced after 1, 5 and 30 minutes of incubation. 2h and 24h, reaction products after 2 and 24h of incubation. Peptides of about 6-7kD were detected within one minute of incubation of the precursor with the enzyme. After 24h only peptides of 6-7kD were detected. The bands smaller than 42kD in track 1 are due to truncated forms of the precursor produced by premature termination of translation in the baculovirus expression system.

Figure 12 Preparative chromatography by reversed phase HPLC of the peptides produced from the precursor by Asp-N digestion

HPLC profile of peptides produced by Asp-N digestion of the PI precursor. The major peaks had a retention time of 19 min (termed Asp-N1) and 21 min (termed Asp-N2). The peptides in these peak fractions (1 & 2) had a slightly slower mobility on SDS-PAGE than the 6kD peptides from stigmas (C, inset). The proteinase inhibitory activity of Asp-N1 and Asp-N2 was tested against trypsin and chymotrypsin.

Figure 13 shows a comparison of the trypsin and chymotrypsin inhibition activity of the PI precursor, PI peptides from stigmas and *in vitro* produced PI peptides from the PI precursor.

PI precursor or PI peptides (0-1.0μg) were tested for their ability to inhibit 1.0μg of trypsin or chymotrypsin as described in the materials and methods. Inhibitory activity is expressed as the percentage of proteinase activity remaining after the proteinase had been preincubated with the PI with 100% remaining activity taken as the activity of the proteinase preincubated with no PI. Experiments were performed in duplicate and mean values were plotted. Deviation from the mean was 8% or less.

Figure 14 is a graphical representation showing a growth curve for T. *commodus* nymphs reared on control artificial diet, soybean Bowman-Birk inhibitor and *N. alata* PI. The vertical axis represents the mean weight of the crickets in each treatment (+/- standard error) in mg. The horizontal axis represents the week number. The crickets reared on the *N. alata* PI showed a lower mean weight than those reared on both the control diet and the diet containing the soybean inhibitor, throughout the experiment.

SUMMARY OF SEQ ID NOs

SEQ ID NO. 1	Nucleotide coding region of *N. alata* PI precursor
SEQ ID NO. 2	Full length nucleotide sequence of *N. alata* PI precursor
SEQ ID NO. 3	Amino acid sequence corresponding to SEQ ID NO. 1
SEQ ID NO. 4	Residues 1-24 of SEQ ID NO. 2 (peptide 1)
SEQ ID NO. 5	Residues 25-82 of SEQ ID NO. 2 (peptide 2)
SEQ ID NO. 6	Residues 83-140 of SEQ ID NO. 2 (peptide 3)
SEQ ID NO. 7	Residues 141-198 of SEQ ID NO. 2 (peptide 4)
SEQ ID NO. 8	Residues 199-256 of SEQ ID NO. 2 (peptide 5)
SEQ ID NO. 9	Residues 257-314 of SEQ ID NO. 2 (peptide 6)
SEQ ID NO. 10	Residues 315-368 of SEQ ID NO. 2 (peptide 7)
SEQ ID NO. 11	N-terminal amino acid sequence of 6kD PI protein
SEQ ID NO. 12	N-terminal amino acid sequence of 6kD PI protein

EXAMPLE 1

1. MATERIALS AND METHODS

Plant Material

Nicotiana alata (Link et Otto) plants of self-incompatibility genotype S_1S_3, S_3S_3 and S_6S_6 were maintained under standard glasshouse conditions as previously described (Anderson *et al*, 1989). Organs were collected directly into liquid Nitrogen to avoid induction of a wound response and stored at -70° until required. To study the effect of wounding on gene expression, leaves were wounded by crushing across the mid-vein with a dialysis clip. Leaves were collected 4 and 24 hours after wounding.

Identification and sequencing of a cDNA clone encoding PI

Polyadenylated RNA was prepared from stigmas and styles, isolated from mature flowers of *N. alata* (genotype S_3S_3), and used to construct a cDNA library in Lambda gt10 (Anderson *et al*, 1989). Single stranded ^{32}P-labelled cDNA was prepared from mRNA from stigmas and styles of *N. alata* (genotype S_3S_3 and S_6S_6) and used to screen the library for highly expressed clones which were not S-genotype specific (Anderson *et al*, 1989). Plaques which hybridised strongly to cDNA probes from both S-genotypes were selected and assembled into groups on the basis of cross-hybridisation. The longest clone of each group was subcloned into M13mp18 and pGEM 3zf+, and sequenced using an Applied Biosystems Model 373A automated sequencer. Both dye primer and dye terminator cycle sequencing chemistries were performed according to standard Applied Biosystems protocols. Consensus sequences were generated using SeqEd™ sequence editing software (Applied Biosystems). The GenBank database was searched for sequences homologous to these clones. Because of the high degree of sequence similarity between the six domains of the *N. alata* PI clone, sequencing primers were made to non-repeated 3' sequences (nucleotides 1117-1137, 1188-1203 and 1247-1267), and to a 5' sequence before the start of the repetitive regions (nucleotides 74-98). In addition, the pNA-PI-2 insert was restricted with endonuclease *Hae*III, which cut at nucleotides 622 and 970 to produce three fragments. The fragments were subcloned into pGEM7zf+ and sequenced in both directions, using the M13 forward and reverse primers. The repetitive nature of the pNA-PI-2 insert rendered it unstable in both phagemid and plasmid vectors when cultures were grown longer than 6 hours.

RNA Gel Blot Analysis

Total RNA was isolated and separated on a 1.2% w/v agarose/formaldehyde gel as previous described (Anderson *et al*, 1989). The RNA was transferred to Hybond-N (Amersham) and probed with the insert from pNA-PI-2 labelled with ^{32}P using random hexanucleotides (1 x 10^8 cpm μg^{-1}; 1 x 10^7 cpm ml^{-1})(Feinberg and Vogelstein, 1983). Prehybridisation and hybridisation, at 68 °C, were as described by Anderson *et al* (1989). The filters were washed in 2 x SSC, 0.1% w/v SDS or 0.2

x SSC, 1% w/v SDS at 68 °C.

***In situ* hybridisation**

In situ hybridisation was performed as described by Cornish *et al*, 1987. The probe was prepared by labelling the insert from pNA-PI-2 (100ng) to a specific activity of 10^8 cpm μg^{-1} by random hexanucleotide priming (Feinberg and Vogelstein, 1983). The labelled probe was precipitated, and resuspended in hybridisation buffer (50µl), and 5µl was applied to the sections. The sections were covered with coverslips, and incubated overnight at 40 °C in a closed box containing 50% v/v formamide. After incubation, sections were washed sequentially in 4 x SSC at room temperature, 2 x SSC at room temperature, and 1 x SSC at 40 °C for 40 min. The slides were dried and exposed directly to X-ray film (Cronex MRF 32, Dupont) at room temperature, overnight. Hybridised sections were counterstained with 0.025% w/v toluidine blue in H_2O, and mounted in Eukitt (Carl Zeiss, Freilburg, FRG). Autoradiographs were transposed over sections to give the composites shown.

DNA Gel Blot Analysis

Genomic DNA was isolated from young leaves of *N. alata* by the procedure of Bernatzky and Tanksley (1986). DNA (10µg) was digested to completion with the restriction endonucleases *Eco*RI or *Hin*dIII, separated by electrophoresis on a 0.9% w/v agarose gel, and transferred to Hybond-N (Amersham) by wet blotting in 20 x SSC. Filters were probed and washed as described for RNA blot analysis.

Preparation of protein extracts

Soluble proteins were extracted from plant material by freezing the tissue in liquid N_2, and grinding to a fine powder in a mortar and pestle. The powdered tissue was extracted in a buffer consisting of 100mM Tris-HCl, pH 8.5, 10mM EDTA, 2mM $CaCl_2$, 14µM β-mercaptoethanol. Insoluble material was removed by centrifugation at 10,000g for 15 min. Protein concentrations were estimated by the method of Bradford (1976) with Bovine Serum Albumin (BSA) as a standard.

Proteinase inhibition assays

Protein extracts and purified protein were assayed for inhibitory activity against trypsin and chymotrypsin as described by Rickauer *et al.* (1989). Inhibitory activity was measured against 1 µg of trypsin (TPCK-treated; Sigma) or 3 µg of chymotrypsin (TLCK-treated; Sigma). The rate of hydrolysis of synthetic substrates N-α-P-tosyl-L-arginine methyl ester (TAME) and N-benzoyl-L-tyrosine ethyl ester (BTEE) by trypsin and chymotrypsin, respectively, were taken as the uninhibited activity of the enzymes. Inhibitory activity of the extract was expressed as the percentage of control protease activity remaining after the protease had been pre-incubated with the extract. The PI peptides from stigma, PI precursor and Asp-N processed peptides were assayed for inhibitory activity as described by Christeller *et al* (1989).

Purification of the *N. alata* PI protein

Stigmas (1000; 10g) were ground to a fine powder in liquid N_2, and extracted in buffer (100mM Tris-HCl, pH8.5, 10mM EDTA, 2mM $CaCl_2$, 14µM β-mercaptoethanol, 4ml/g tissue). To concentrate the extract prior to the first purification step, gel filtration, the inhibitory activity was precipitated with 80% w/v ammonium sulphate, the concentration required to precipitate all the proteinase inhibitory activity.

The ammonium sulphate pellet was resuspended in 5ml of 0.15M KCl, 10mM Tris-HCl, pH 8.1, and loaded onto a Sephadex G-50 column (2cm x 100cm) equilibrated with the same buffer. The fractions (10ml) eluted from this column and containing proteinase inhibitory activity were pooled and applied to an affinity column of Chymotrypsin-Sepharose CL4B [100mg TLCK-treated α-chymotrypsin (Sigma) cross-linked to 15ml Sepharose CL4B (Pharmacia) by manufacturers instructions]. The column was washed with 10 volumes of 0.15M KCl/10mM Tris-HCl, pH 8.1, prior to elution of bound proteins with 7m urea, pH 3 (5 ml fractions). The eluate was neutralised immediately with 200 µl 1M Tris-HCl pH 8, and dialyzed extensively against deionised H_2O.

Amino acid sequence analysis

Purified PI protein was chromatographed on a reverse phase HPLC microbore column prior to automated Edman degradation on a gas phase sequencer (Mau *et al*, 1986). Phenylthiohydantoin (PTH) amino acids were analysed by HPLC as described by Grego *et al* (1985).

Production of a polyclonal antiserum to the *N. alata* PI

The purified proteinase inhibitor (Figure 6c, lane 3) was conjugated to a carrier protein, keyhole limpet haemocyanin (KLH) (Sigma), using glutaraldehyde, as follows. 1mg of PI protein was dissolved in 1.5ml H_2O, and mixed with 0.3 mg KLH in 0.5 ml of 0.4M phosphate buffer, pH7.5. 1ml of 20mM glutaraldehyde was added dropwise over 5 min, with stirring at room temperature. The mixture was stirred for 30 min at room temperature, 0.25ml of glycine was added, and the mixture was stirred for a further 30 min. The conjugated protein was then dialyzed extensively against normal saline (0.8% w/v NaCl). The equivalent of 100µg of PI protein was used for each injection. Freund's complete adjuvant was used for the first injection, and incomplete adjuvant for two subsequent booster injections. The IgG fraction of the antiserum was separated on Protein A Sepharose (Pharmacia) according to manufacturer's instructions.

Protein Gel Blot Analysis

Protein extracts were electrophoresed in 15% w/v SDS-polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose in 25mM Tris-HCl, 192mM glycine, 20% v/v methanol, using a BioRad Trans-Blot^{R}Semi-dry electrophoretic transfer cell (12V, 20 min). Loading and protein transfer were checked by staining the proteins on the membranes with Ponceau S (Harlow and Lane, 1988). Membranes were blocked in 3% w/v bovine serum albumin for 1h, and incubated with the anti-PI antibody (2µg/ml in 1% w/v BSA, Tris Buffered Saline) overnight at room temperature. Bound antibody was detected using biotinylated donkey anti-rabbit IgG (1/500 dilution, Amersham) and the Amersham Biotin-Streptavidin system according to procedures recommended by the manufacturer.

Proteolysis of the PI precursor by endoproteinase Asp-N

Affinity-purified PI precursor (1.25mg) was incubated at 37°C with endoproteinase Asp-N (2µg) in 100mM NH_4HCO_3, pH 8.5 in a total volume of 1ml for 48h. Reaction products were separated by reversed-phase HPLC using an analytical Brownlee RP-300 Aquapore column (C8, 7µm, 4.6x100mm). The column was equilibrated in 0.1% v/v TFA and peptides were eluted with the following program: 0-25%B (60% v/v acetonitrile in 0.089% v/v TFA) applied over 5min, followed by a gradient of 25-42%B over the next 40min, and ending with a gradient of 42-100%B over 5 minutes. The flow rate was 1.0ml/min and peptides were detected by absorbance at 215nm. Each peak was collected manually and freeze dried. Concentration was estimated by response obtained with each peak on the UV detector at 215nm.

2. CLONING OF PI PRECURSOR GENE

Isolation and characterisation of the PI cDNA clone

A cDNA library, prepared from mRNA isolated from the stigmas and styles of mature flowers of *N. alata*, was screened for clones of highly expressed genes which were not associated with self-incompatibility genotype. Clones encoding a protein with some sequence identity to the type II proteinase inhibitors from potato and tomato (Thornburg *et al*, 1987; Graham *et al*, 1985) were selected. The largest clone, NA-PI-2, is 1360 base pairs long with an open reading frame of 1191 nucleotides. The nucleic acid sequence (SEQ ID NO. 2) and the predicted amino acid sequence (SEQ ID NO. 3) of the *N. alata* clone, NA-PI-2 is shown in Figure 1. There are no potential N-glycosylation sites.

Surprisingly, the *N. alata* cDNA clone encodes a protein with six repeated domains that have high, but not perfect, sequence identity (Figure 1). Each of these domains contains a potential reactive site which is highlighted in Figure 1. The residues at the putative reactives sites of the *N. alata* PI are consistent with the inhibitor having two sites which would specifically inhibit chymotrypsin (Leu5-Asn6, Leu63-Asn64) and four sites specific for trypsin (Arg121-Asn122, Arg179-Asn180, Arg237-Asn238

and Arg295-Asn296).

To ensure that the repeat structure of NA-PI-2 was not due to a cloning artifact, three additional cDNA clones were sequenced, and found to be identical to NA-PI-2.

Table 1 is a comparison of the percentage amino acid identity of the six domains of the PI precursor.

Temporal and spatial expression of the PI mRNA

Total RNA, isolated from various tissues of *N. alata*, was probed with the PI cDNA clone in the RNA gel blot analyses shown in Figure 2. Two hybridising messages of 1.0 and 1.4kb were present in total RNA isolated from styles (including stigmas). Only the larger message, which was predominant in this tissue, is of sufficient size to encode the cDNA clone NA-PI-2 (1.4kb). The smaller message is not detected with the cDNA probe at higher stringency. An homologous message of approximately 1.4kb was also present in RNA isolated from the styles of *N. tabacum* and *N. sylvestris* (Figure 2).

In the other floral organs (except pollen), both messages were detectable at low levels, however, the smaller RNA species appeared more abundant. There was no hybridisation to pollen RNA. No hybridising species were evident in leaf RNA, but two species, 1.0 and 1.4kb were detected 24 hours after mechanical wounding. The smaller message (1.0kb) was more abundant in this case.

In situ hybridisation of radiolabelled *N. alata* PI cDNA to longitudinal sections of styles from immature (1cm long) buds is shown in Figure 3. RNA homologous to the cDNA clone bound strongly to cells of the stigma and weakly to vascular bundles. No hybridisation was detected in the cortical tissue, transmitting tract tissue, or epidermis of the style. The same pattern of hybridisation was observed in mature receptive flowers. Control sections treated with ribonuclease A prior to hybridisation were not labelled.

Genomic DNA blot analysis

The cDNA clone NA-PI-2, was used as a probe on the DNA gel blot shown in Figure 4 which contained genomic DNA, digested with either *Eco*RI or *Hin*dIII. *Eco*RI produced two hybridising fragments (11kb and 7.8kb) and *Hin*dIII produced three large hybridising fragments (16.6, 13.5 and 10.5 kb).

Distribution of PI activity in various tissues of *N. alata*

The inhibition of trypsin and chymotrypsin by crude extracts of various organs of *N. alata* is shown in Figure 5. Stigma extract was the most effective inhibitor of both trypsin and chymotrypsin. The stigma extracts had up to eight times more inhibitory activity than sepal extracts, and more than 20 times more activity than extracts from styles, petals, leaves and wounded leaves.

Purification of PI from *N. alata* stigmas

Stigmas of *N. alata* were extracted in buffer and the inhibitory activity was concentrated by precipitation with 80% w/v ammonium sulphate. The precipitate was redissolved and fractionated by gel filtration on Sephadex G-50. Most of the protein in the extract eluted early in the profile illustrated in Figures 6a and 6b, relative to the proteinase inhibitor. Fractions with proteinase inhibitor activity were pooled and applied to an affinity column of chymotrypsin-Sepharose. The PI activity co-eluted with a protein of about 6kD, which appeared to migrate as a single band on the 20% SDS-polyacrylamide gel shown in Figure 6c. The purity of the PI at various stages of purification was assessed by SDS-PAGE (Figure 6c). The purified inhibitor represented approximately 50% of the inhibitory activity present in the crude extract.

Amino acid sequence of the N-terminus of the 6kD PI protein

The N-terminal amino acid sequence DRICTNCCAG(T/K)KG (SEQ ID NO. 11; SEQ ID NO. 12, respectively) was obtained from the purified PI protein. This sequence of amino acids corresponds to six regions in the deduced sequence of the cDNA clone, starting at positions 25, 83, 141, 199, 257 and 315 in Figure 1. At position 11 of the N-terminal sequence, both threonine and lysine were detected.

This is consistent with the purified inhibitor comprising a mixture of six peptides beginning with the sequences underlined in Figure 1, as the first two peptides contain threonine at this position, while the other four peptides have lysine at this position. The position of these peptides relative to the six repeated domains in the predicted precursor protein is illustrated in Figure 7. Five of the six predicted 6kD peptides, contain a reactive site for either chymotrypsin or trypsin (Figure 1 and 7). The sixth potential peptide is four amino-acids shorter than the other five peptides (fifty eight amino-acids) and may not be active, as it does not contain an inhibitory site. The peptide from the N-terminus (x in Figure 7) has a potential chymotrypsin reactive site but is much shorter (24 amino acids).

Distribution of the PI protein in *N. alata*

A polyclonal antiserum was raised to the purified PI protein conjugated to keyhole limpet haemocyanin. The antibody reacted strongly with the purified 6kD PI protein in immunoblot analyses and bound only to a 6kD and a 32kD protein, which appears as a doublet, in total stigma and style extracts from mature flowers. Figure 8 is an immunoblot containing protein extracts of stigmas from flowers at different stages of development (1cm long buds to mature flowers) probed with the anti-PI antiserum. Larger cross reacting proteins of approximately 18kD, and 42kD were detected in buds from 1cm to 5cm in length in addition to the 6kD and the 32kD protein. The 18kD and 42kD proteins decreased in concentration with maturity, while the 6kD protein reached a peak concentration just before anthesis. The concentration of the 32kD protein remained relatively constant during flower maturation.

TABLE 1

N. alata PI							
		1	2	3	4	5	6
N. alata	1		100	88	88	90	79
	2			88	88	90	79
	3				97	95	86
	4					98	90
	5						90
	6						

EXAMPLE 2
PURIFICATION AND IDENTIFICATION OF PI MONOMERS
1. MATERIALS AND METHODS

Separation of the 6kD PI species by reversed phase chromatography

Stigmas (21,000) were ground and extracted as described for purification of the PI protein. After gel filtration on a Sephadex G-50 gel filtration column (5cmx800cm, 3000 stigmas per separation) the peptides were lyophilized and applied to a Brownlee RP-300 C8 Reversed-phase column, 10x250mm, on a Beckman HPLC system Gold, and eluted with 0.1% v/v Trifluoroacetic acid (TFA) and an acetonitrile gradient (0-10% over 5mins, 10-25% over 40 mins and 25-60% over 10 mins), at 5ml/min. Peak fractions, designated fraction 1, 2, 3 and 4 were collected and freeze dried.

Electrospray mass spectrometry

On line mass spectrometric analysis of HPLC eluates was performed by application of 20 pmoles of each PI preparation (fraction 1, 2, 3 & 4) in 2µl of water onto a Brownlee RP-300 C8 reversed-phase column (150x0.20mm internal diameter fused-silica capillary column) on a modified Hewlett- Packard model HP1090L liquid chromatograph and elution with a linear gradient of acetonitrile (0.05% v/v TFA to 0.045% v/v TFA/60% v/v acetonitrile in 30 min.) at a flow rate of 1µl/min and a column temperature of 25°C. The eluant was monitored at 215nm using a Spectral Physics forward optics scanning detector with a 6-mm pathlength U-shaped axial beam capillary flow cell (LC Packings, Netherlands). Mass spectra were acquired on a Finnigan-Mat triple quadrupole mass spectrometer (modelTSQ-700, San Jose, CA) equipped with an electrospray ionisation (ESI) source (Analytica, Branford, CT). The electrospray needle was operated in positive ion mode at a voltage differential of -4 kV. The sheath liquid was 2-methoxyethanol delivered at 1µl/min via a syringe drive (Harvard Apparatus, South Natick, MA). The nitrogen drying gas conditions were as follows: heater temperature, 275°C; pressure, 15 psi; flow rate, ~ 15stdL/min. The nitrogen sheath gas was supplied at 33 psi. Gaseous nitrogen was

obtained from a boiling liquid nitrogen source. Peptides were introduced into the ESI source at 1.0 µl/min by on-line capillary RP-HPLC as described above. Spectra were acquired scanning from *m/z* 400 to 2000 at a rate of 3sec. Data collection and reduction were performed on a Dec5100 computer using Finnigan BIOMASS™ software.

2. RESULTS

Separation and identification of the individual 6kD PI species from *N.alata* stigmas
The five of the six peptides of about 6kD that were predicted to be present in the purified 6kD PI preparation have been separated from each other by reversed-phase HPLC chromatography. Four peaks were obtained (Fig. 9a) and the peptides within each peak were identified by electrospray mass spectrometry (Table 2). The peptides have been designated C1, T1, T2, T3 and T4 according to their position in the PI precursor and the presence of a chymotrypsin or trypsin reactive site (Fig 9b). The first HPLC peak (Fig 9a) corresponds to the chymotrypsin inhibitor C1, the second peak is composed of a mixture of T2 and T3 (identical to each other) and T4 that differs from T2 and T3 by one amino-acid at position 32. The third peak contains the peptide T1 and the fourth peak is composed of a mixture of T1, T2/T3 and T4 (Table 2).

The site of processing has not been precisely determined, but is likely to be located between the aspartate (N) and asparagine (D) residues in the sequence outlined in Figure 10. Proteases with specific requirements for asparagine residues have been isolated from vacuoles from immature soybean seeds and pumpkin cotyledons (Scott et al., 1992, Hara-Nishimura et al., 1991). This is consistent with the immunogold localization of the PI in the vacuoles of the papillae and the underlying secretory cells in the stigma of *N.alata* (Atkinson, 1992). In the case of the *N.alata* PI, processing analogous to that of peptide hormones is also possible because each of the possible 6kD peptides are flanked by dibasic residues (Lys-Lys, position -2 &-3 in Figure 10). However, a system like this has not been described in plants, and it is more likely that the dibasic residues contribute to the predicted hydrophilic loops

that present the processing site on the surface of the molecule.

The data from the mass spectrometric analysis shows that once the initial cleavage has occurred the new carboxy terminus is trimmed back (Figure 10). The EEKKN sequence (SEQ ID NO. 14) is removed completely but the trimming is not precise, sometimes an additional amino acid is removed. Steric hindrance probably prevents further trimming. Occasionally the aspartate is also removed from the N-terminus.

EXAMPLE 3

Production of PI precursor in insect cell (Sf9) culture using a recombinant baculovirus vector.

cDNA encoding the PI precursor (Figure 1) was inserted into the Eco R1 site of the plasmid vector pVL 1392, which is the same as pVL941 (Lucknow and Summers, 1989) except that a multiple cloning site was inserted at the BamH1 site. The plasmid designated pRH11, contains the PI cDNA in the correct orientation with respect to the direction of transcription directed by the polyhedrin promoter. Recombinant baculovirus was obtained by co-transfection of *Spodoptera frugiperda* cells with baculovirus DNA and pRH11. The recombinant viruses, produced by homologous recombination, were plaque purified and amplified prior to infection of insect cells for protein production. All procedures for production of recombinant baculovirus, titration of the virus and maintenance and infection of the Sf9 cells were obtained from King and Posse (1992). For production of the PI precursor, monolayers of Sf9 cells in large flasks (175cm^2) were infected at the time of confluence with an inoculum of high-titre recombinant virus at a multiplicity of infection of 5-10 pfu/cell. Culture fluid was collected after 4 days of infection, clarified by centrifugation and the PI precursor was purified by application to a Chymotrypsin-Sepharose affinity column as described for the 6kD PI species from stigmas. PI precursor eluted from the column in 7M urea, pH3 was neutralized immediately with 1M Tris-HCl buffer pH8, dialysed extensively against Milli-Q water, concentrated 20-50 fold by ultrafiltration using a Diaflow YM10 filter and stored frozen at -20°C.

The cDNA clone encoding the PI precursor was engineered into a baculovuirus vector for the production of the precursor from infected insect cells. The insect cells produced a 42kD protein that cross reacted with the antibodies raised to the 6kD PI peptides from stigma and bound to the chymotrypsin affinity column. This 42kD protein was identical in size to the 42kD precursor produced in the immature stigmas of *N.alata* (Fig.11) and had the N-terminal sequence LysAlaCysThrLeuAsn (SEQ ID NO. 13) demonstrating that the signal sequence had been processed correctly by the insect cells (Fig.1). Based on these results, the 42kD protein produced in the baculovirus expression system will now be referred to as the PI precursor. The 42kD PI precursor had inhibitory activity against chymotrypsin but no inhibitory activity against trypsin (Fig.13). Processing of the PI precursor by the endoproteinase AspN led to the production of stable peptides of about 6kD that were partially purified by reversed phase HPLC (Fig.12). These peptides have equivalent inhibitory activity against trypsin and chymotrypsin as the 6kD peptides isolated from stigma, indicating that processing of the precursor is required to activate the trypsin inhibitory activity but not all the chymotrypsin activity. Since AspN cleaves specifically adjacent to Aspartate residues (between Asn-1 and Asp1 in Figure 10) and has no trimming activity, the peptides produced *in vitro* will be similiar to those produced in stigmas except for the presence of the sequence EEKKN (SEQ ID NO. 14) at the C-terminus. This provides further evidence that precise processing of the N-and C-termini is not required to obtain an active 6kD PI peptide. Asp-N1 is more efficient at inhibiting chymotrypsin than trypsin and is thus likely to be predominantly a C1 analogue (Fig.9b). Asp-N2 is a more efficient trypsin inhibitor and probably contains the T1-T4 analogues.

EXAMPLE 4

Effect of PIs on protease activity in unfractionated gut extracts from various insects
Activity of PIs on gut proteases was measured using the procedure of Christeller et al., (1992) as follows. An aliquot of 1uM of inhibitor (0-10µl, at least 5-fold excess over proteases present in the gut) was mixed with 150µl of 10mM CAPS buffer, pH 10, and preincubated with each insect gut extract (0-15µl), for 20 min at 30°C. The reaction was started by the addition of 50µl of ^{14}C -labelled casein substrate (400µg protein, specific activity 25,000-75,000 dpm mg^{-1}) and continued for 30 min at 30°C until 50µl of cold 30% (w/v) TCA was added to terminate the reaction. After incubation on ice for 30 min, undigested protein was pelleted by centrifugation at 20°C for 5 min at 10,000g. The supernatant was removed, mixed with scintillation fluid and the radioactivity measured. Assays were performed at pH 10 except for *L.sericata* and *C.rufifacies* when 10mM Tris-HCl, pH 8.0 was used.

Table 3 shows the inhibitory activity of the pooled 6kD PI peptides (C1, T1, T2/T3, T4), the mixture of trypsin inhibitors T2/T3 and T4, and the chymotrypsin inhibitor C1 against the proteases in the gut of various members of the Lepidoptera, Coleoptera, Orthoptera and Diptera. In most cases, the pooled peptides and the trypsin inhibitors had an equivalent effect against the gut proteases with the degree of inhibition ranging from 37-79% depending on the insect tested. The inhibitors had negligible effect on the gut proteases of the potato tuber moth, *P.operculella*. The chymotrypsin inhibitor C1 also affected the activity of the proteases but was less effective than the trypsin inhibitors in five cases (*W.cervinata, L.serricata, C.zealandica, P.octo*, sugar cane grub).

The experimental details are described in the legend to Figure 14. The *N. alata* PI was more effective than Soybean Bowman-Birk inhibitor in reducing cricket weight. It has shown that there is a good correlation between the ability of a proteinase inhibitor to inhibit the enzymes of the insect midgut and its effectiveness in retarding the growth of insects in insect feeding trials (Christeller et al., 1992). Figure 14

shows that the pooled PIs that inhibited the gut proteases of the black field cricket (T.commodus) by 70% in the *in vitro* assay retarded the growth of the crickets by 30% in a feeding trial conducted over a 10 week period. The correlation between *in vitro* assays and feeding trials has been confirmed recently by Johnston and collegues (1993) working on growth and development of *Helicoverpa armigera*.

TABLE 2

HPLC peak	retention time (min)	molecular weight	assigned peptide*
1	15.5	5731.5	C1
		5644.4	C1 minus Ser_{53}
		5616.4	C1 minus Asp_1 & Ser_{53}
		55.29.3	C1 minus Asp_1
2	20.5	5700.5	T2/T3
		5728.5	T4
		5585.4	T2/T3 minus Asp_1
		5613.5	T4 minus Asp_1
3	22.5	5725.5	T1
		5610.5	T1 minus Asp_1
4	24	5654.4	T1 minus Ala_{53}
		5641.4	T4 minus Ser_{53}
		5613.4	T2/T3 minus Ser_{53}
		5539.4	T1 minus Asp_1 & Ala_{53}
		5498.4	T2/T3 minus Asp_1 & Ser_{53}
		5526.4	T4 minus Asp_1 & Ser_{53}

* See Figure 9 for designation of C1 and T1-T4.

TABLE 3

Effect of *Nicotiana alata* proteinase inhibitors and Potato inhibitor II on casein hydrolysis by crude gut extracts

	casein hydrolysis (% control)		
Insect	**NaPI**	**C1**	**T2/T3, T4**
H. armigera	33.2	32.7	30.3
H. punctigera	26.6	29.3	28.5
T. commodus	28.4	35.0	33.1
A. infusa	37.5	40.2	43.3
sugar cane grub	25.8	43.9	25.1
W. cervinata	22.9	82.9	20.4
E. postvitiana	39.7	45.4	41.2
S. litura	28.1	33.6	24.8
P. operculella	95.8	100	98.5
C. rufifacies	29.1	37.8	28.9
L. serricata	59.2	100	63.0
C. zealandica	31.7	54.7	32.0
P. octo	57.1	67.2	57.4
C. obliquana	51.1	49.1	45.5
A. tasmaniae	28.3	34.2	39.5

Legend to Table 3

NaPI = *N. alata* proteinase inhibitors pooled

C1 = *N. alata* chymotrypsin inhibitor (peak 1 from HPLC)

T2/T3, T4 = *N. alata* trypsin inhibitors (peak 2 from HPLC)

Heliothis armigera, Helicoverpa armigera, Tobacco budworm, Lepidoptera

Heliothis punctigera, Helicoverpa punctigera Native budworm, Lepidoptera

Teleogryllus commodus Black field cricket, Orthoptera

Agrotis infusa Common cutworm, adults known as the Bogong moth, Lepidoptera

Wiseana cervinata Porina, native to New Zealand, Lepidoptera

Lucilla sericata Green blow fly, Diptera, assayed at pH8

Chrysomya rufifacies Hairy maggot blow fly, Diptera, assayed at pH8

Aphodius tasmaniae Tasmanian grass grub = Black-headed pasture cockchafer, Coleoptera

Costelytra zealandica New Zealand grass grub, Coleoptera

Spodoptera litura Tropical armyworm, Lepidoptera

Phthorimaea operculella Potato tuber moth, Lepidoptera

Epiphyas postvittana Lightbrown apple moth (leafroller), Lepidoptera

Planotortrix octo Greenheaded leafroller, Lepidoptera

Ctenopseustis obliquana Brownheaded leafroller, Lepidoptera

Sugar cane grub

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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